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IMMOBILIZED SILVER IMMUNOASSAY SYSTEM

Specification

Background of the Invention

Since the late 1950s, when the work of Rosalyn Yalow and Solomon Berson (Yalow et al., 1959, Nature (London) 184:1648; Yalow et al., 1960, J. Clin. Invest 39:1157) first illuminated the possibilities of immunoassays, immunodiagnostic techniques based on the specific interaction of antibody and antigen have become of paramount importance in the clinical, agricultural, food, veterinary, and environmental sectors. In 1996, it was estimated that the worldwide market of immunoassay products was $10 billion in the clinical sector alone, and increasing at an annual rate of 10% (Deshpande, Enzyme Immunoassay: From Concept to Product Development, New York:Chapman & Hall, 1996). This market is driven by an ever-increasing desire for assays of greater sensitivity and specificity, at reasonable financial costs. It is in this environment that the specific and irreversible interaction between avidin or streptavidin and biotin has found use.

Streptavidin, a close relative of egg white avidin, is expressed in *Streptomyces avidinii* (Green, 1990, Methods in Enzymology 184:51), and both avidin and streptavidin exhibit an affinity for biotin on the order of 10^{15} M^{-1}. The streptavidin-biotin system has become a widely-used tool of molecular biology in such applications as affinity chromatography, cytometry, nucleic acid research, and diagnostics (Diamandis et al., 1991, Clin. Chem. 37:625; Wilchek et al., 1988, Anal. Bio Chem. 171:1). A common immunological procedure calls for the use of streptavidin-coated microtiter plates, which are used to capture either biotinylated antibodies or antigens. Since the assay is based on the interaction of streptavidin and biotin, universal kit-based assay formats are possible. These universal assays are also the basis of many automated immunological testing systems (Chan, ed., 1996, Immunoassay Automation: An Updated Guide to Systems, San Diego: Academic Press, 51-308). A format utilizing a microtiter-based enzyme-linked immunosorbent

assay (ELISA) can measure a wide variety of analytes using a dual antibody or "sandwich" immunoassay. The choice of a streptavidin-coated solid support is made to overcome the limitations present in the direct antibody coating of polystyrene supports, which can result in unreliable or nonuniform coating of the solid support, in addition to the steric affects of binding upon the antibody. However, while streptavidin systems allow for universal ELISA kits and can improve assay sensitivity, coated plates can be costly.

Research examining the behavior of biotin has found that immobilized silver ions will bind biotinylated compounds both strongly and, in this case, reversibly. This research has been done with both immobilized metal affinity chromatography (IMAC) (Garcia et al., 1994, Reactive Polymers 23:249; Kim et al., 1995, Art. Cells, Blood Subs., and Immob. Biotech. 23:555; Miles et al., 1995, J. Chromatogr. A. 702:173) and paramagnetic particles (Ramirez-Vick, 1997, Ph.D. Dissertation, Arizona State University, Tempe, AZ). It has been discovered in accordance with the present invention that immobilized silver ions can be used in an immunoassay format to provide a sensitive and inexpensive universal assay.

Summary of the Invention

The present invention provides an immunoassay system comprising bioassay plates having silver immobilized thereon. The present invention further provides a method of making bioassay plates having silver immobilized thereon.

In another embodiment, the present invention provides a method for detecting an antigen or antibody, and a kit useful for the detection of an antigen or antibody.

An apparatus for providing activated bioassay plates is also provided by the present invention.

Brief Description of the Drawings

Fig. 1 is a graph depicting the sensitivity of a microtiter plate having silver immobilized thereon.

Fig. 2 is a schematic of an immunoassay utilizing microtiter plates having silver immobilized thereon, and biotinylated capture antibodies.

Fig. 3 is a graph providing a kinetic analysis of a horseradish peroxidase immunoassay.

Fig. 4 is a schematic of a checkerboard assay.

Fig. 5 shows the results of a checkerboard assay.

Fig. 6 is a schematic depicting the arrangement of activated and control wells of a microtiter plate.

Fig. 7 is a graph of immunoassay results comparing a streptavidin method with the silver method of the present invention.

Fig. 8 is a flow diagram describing the apparatus of the present invention.

Fig. 9 is a side view of the apparatus of the invention.

Fig. 10 is a diagram of the liquid handling system of the invention.

Figs. 11a-11c are diagrams of the liquid transfer manifold of the invention. Fig. 11a is a side view of the reagent addition stage and vacuum stage; Fig. 11b is a front view of the reagent addition stage; Fig. 11c is a front view of the vacuum stage.

Detailed Description of the Invention

The present invention provides an immunoassay system comprising bioassay plates having silver, in particular silver ions, immobilized thereon. The invention further provides methods of making and using such bioassay plates. The bioassay plates and immunoassay of the present invention are useful for the detection of antibodies and antigens, and provide cost and sensitivity advantages relative to the streptavidin-coated bioassay plates of the prior art.

The bioassay plates used in the present invention are microwell, or microtiter, plates known in the art for immunoassays, and are commercially available. Conventional microwell plates are 96-well microplates having wells arranged on an 8 x 12 matrix on 9 mm centers. Each well holds approximately 300 microliters. 384-well plates are also available, in which the wells are arranged in a 16 x 24 matrix on a 4.5 mm center, with each wells having a brim volume of approximately 80 microliters. Well plates defined by larger matrices, e.g. 1536 well plates, are also available. The number and configuration of the wells are not critical to the present

invention, and are used by way of example only. The bioassay plates used in accordance with the present invention are plastic, and preferably polystyrene.

Bioassay plates having silver ions immobilized thereon are made by a method comprising functionalizing a multi-well bioassay plate to provide an amine-containing bioassay plate, adding polymerized glutaraldehyde to the wells of the plate for a time and under conditions whereby the amines are activated by glutaraldehyde, rinsing the plates with an aqueous solution, adding thiourea to the wells of the plate for a time and under conditions whereby the thiourea is reacted with a glutaraldehyde moiety of the glutaraldehyde-activated bioassay plate, rinsing the plate with an aqueous solution, and contacting the plate with silver ions for a time and under conditions whereby the silver ions are immobilized on said plate.

The bioassay plate may be constructed of any material that can be functionalized to contain an amine group. In a preferred embodiment, the multi-well bioassay plate is a plastic multi-well bioassay plate. For example, the plate may be made of polystyrene, polyethylene, polypropylene, or other primary polymers or composite resins. Polystyrene is particularly preferred. Methods for functionalizing these materials to contain an amine group are known in the art. For example, a polystyrene bioassay plate can be functionalized to contain an amine group by methods known in the art and disclosed for example in Immobilized Affinity Ligand Techniques, Hermanson et al., eds., San Diego: Academic Press, 1992, the disclosure of which is incorporated herein by reference. Aminated polystyrene bioassay plates are also commercially available, for example from Corning (Corning, NY), NUNC (Denmark) and Micro Membranes (Newark, NJ). Preferably the plate is amidated or aminated to contain from about 1×10^{13} to 1×10^{14} amine sites per cm^2.

Polymerized glutaraldehyde may be prepared by allowing glutaraldehyde (25 wt %) to polymerize, for example for from 1 to 36 hours at from 23°C to 70°C. In a preferred embodiment polymerization is at 70°C for about 24 hours. The polymerized glutaraldehyde is added to the wells of the plate and incubated under conditions whereby a glutaraldehyde-activated plate is produced, for example for from 1 to 36 hours at from 23°C to 70°C. In a preferred embodiment, incubation is at 35 to 50°C for 1 to 24 hours, and more preferably at about 37°C for about 24 hours. The

plate is rinsed with an aqueous solution, for example deionized water, to remove unreacted glutaraldehyde. The wells of the plate are then filled with thiourea, for example from 0.01M to 1M solution, and preferably a 1M solution, under conditions suitable for reaction with the glutaraldehyde moiety, for example for from 1 to 36 hours at from 23°C to 70°C. In a preferred embodiment at, incubation is for 1 to 24 hours at 35 to 50°C, and more preferably about 24 hours at about 37°C. The plate is rinsed with an aqueous solution, for example deionized water, to remove unreacted thiourea.

Silver ions, preferably in the form of silver nitrate, are then added to the plate under conditions whereby silver ions are immobilized on the plate, for example for from 1 to 36 hours at from 23°C to 70°C. In a preferred embodiment, incubation is for about 24 hours at about 37°C. The plates are then rinsed with an aqueous solution, for example deionized water, and may be stored until use, preferably in an opaque sleeve.

The bioassay plates having silver ions immobilized thereon are useful in a method for detecting an antigen or an antibody. It has been discovered in accordance with the present invention that the silver ions immobilized on bioassay plates are capable of strong binding to biotinylated antibodies and antigens. Accordingly, the plates of the invention may be used in standard enzyme-linked immunosorbent assays (ELISAs). For example, a bioassay plate having silver ions immobilized thereon is incubated with biotinylated antibody to provide a bioassay plate having the antibody immobilized thereon. After washing with an aqueous solution, the plate is incubated with a solution containing the cognate antigen under conditions whereby the antigen binds to the immobilized antibody, followed by another washing step. The antigen is then detected, for example by subsequent incubation with a labeled antibody having specificity for the antigen. Detectable labels for antibodies are known in the art and include radiolabels, fluorescent tags, and enzyme conjugates. In a preferred embodiment, the aqueous solution contains deionized water and Tween (polyoxyethylene sorbitan monolaurate).

An antibody may be detected using the plates of the present invention in an indirect ELISA assay. For example, a bioassay plate having silver ions immobilized

thereon is incubated with a biotinylated antigen to provide a bioassay plate having the antigen immobilized thereon. After washing with an aqueous solution, the plate is incubated with a solution containing the cognate primary antibody under conditions whereby said antibody binds to the immobilized antigen. After incubation and washing, a labeled secondary antibody is added and incubated under conditions whereby it binds to the primary antibody. After washing, the secondary antibody is detected, wherein detection thereof indicates the presence of the primary antibody. Detectable labels for antigens are known in the art and include radiolabels, fluorescent tags, and enzyme conjugates. In a preferred embodiment, the aqueous solution contains deionized water and Tween.

Conditions for biotinylating antibodies and antigens are well known in the art and disclosed, for example, by Bayer et al., "Protein Biotinylation" (1990) Methods in Enzymology 184:138 and O'Shannessy "Antibodies Biotinylated via Sugar Moieties" (1990) Methods in Enzymology 184:162, the disclosures of which are incorporated herein by reference. Conditions for performing ELISAs are well-known in the art and disclosed, for example, by Harlowe, et al., (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, the disclosure of which is incorporated herein by reference.

The present invention further provides a kit useful for the detection of an antigen or antibody. The kit comprises, in a first container, a bioassay plate having silver ions immobilized thereon. In a preferred embodiment, the bioassay plate is a polystyrene multi-well plate. The kit may optionally contain a second container containing a biotinylated antibody or a biotinylated antigen. The kit may optionally contain a third container containing labeled antibody, when the second container contains a biotinylated antibody, or a labeled secondary antibody, when the second container contains a biotinylated antigen.

The present invention further provides an apparatus useful for the automated production of microplates having modified surface chemistry. As shown by the flow diagram in Fig. 8, the apparatus provides for filling the wells of a microplate with a reagent in an addition/withdrawal chamber; conveying the microplate to an incubation chamber in which the microplate is sealed, heated and agitated, and unsealed;

conveying the microplate to the addition/withdrawal chamber for evacuation of reagent, washing, and addition of a second reagent; conveying the microplate to the incubation chamber for sealing, heating and agitation, and unsealing; conveying the microplate to the addition/withdrawal chamber for evacuation of reagent and washing; followed by subsequent cycles of reagent addition and incubation, or conveyance of the microplate out of the machine.

In a preferred embodiment, and with reference to Figs. 9, 10, 11a, 11b and 11c, the apparatus comprises a housing having disposed therein a reagent addition/withdrawal chamber (1) and an incubation chamber (2). Microplates are conveyed into and between the chambers by means of a plate holder (3) movable horizontally by a plate holder track (4). Reagents and wash solution are provided in storage containers (5) connected by reagent lines (13) to the dispense portion of a manifold (6) which delivers reagent and wash solution by dispense lines (7) by means of a liquid pump (11). After reagent addition, microplates positioned on the plate holder (3) are conveyed via the plate holder track (4) into the incubation chamber (2). The microplate is sealed by a non-reactive sealing plate (13) delivered vertically. The incubation chamber further provides a means for heating and agitating the microplate (14). After a time predetermined by the user, the microplate is conveyed to the reagent addition/withdrawal chamber (1) via plate holder track (4). Spent reagent is removed through aspirator lines (8) and withdrawn by the aspirate portion of the same manifold (6) by means of a vacuum pump (9) through waste lines (12) to a waste container (10). Wash solution is added through the dispense portion of the manifold (6) which delivers wash solution through dispense lines (7). Wash solution is removed through aspirator lines (8) and withdrawn by the aspirate portion of manifold (6) by means of a vacuum pump (9) through waste lines (12) to a waste container (10).

The foregoing steps are carried out in an automatic programmed manner under the control of electronic circuitry contained in the housing.

All references cited herein are incorporated in their entirety.

The following examples serve to further illustrate the present invention.

Example 1

A polystyrene 96 well microtiter plate aminated to provide approximately 2 x 10^{13} active amine sites per cm^2 was obtained from Corning (Corning, NY). Glutaraldehyde (25 wt %) which had been allowed to polymerize at 70°C for 24 hours was added to each well of the microplate, which was then incubated at 37°C for 24 hours to facilitate plate activation. The plate was then rinsed with deionized water and wells filled with a 1M solution of thiourea, followed by an additional 24 hour period of incubation at 37°C. After another rinsing of the plate, a 1M solution of silver nitrate was allowed to contact the plate during another 24 hour incubation at 37°C. The plate was then rinsed extensively.

The biotin-binding capability of such an immobilized silver microtiter plate was tested as follows. A complete plate was assembled from stripwells, using alternating strips of unactivated and silver-containing wells, where the silver-containing strips were in the odd-numbered rows and the unactivated strips were in the even-numbered rows. The test consisted of the binding of biotinylated horseradish peroxidase (bHRPO), which was detected using the chromogenic reaction of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS®). This was a one-step, unamplified assay; 150 µl of the bHRPO solution was added to the wells using doubling dilutions, and allowed to bind for one hour. Following this, the plates were rinsed with deionized water and the ABTS® in citrate buffer was added. The developed color in the wells was read after one hour at room temperature using a BioRad Benchmark Microplate Reader set at 415 nm. The plate setup and moles of bHRPO corresponding to each dilution are shown in Tables I and II, respectively.

TABLE I

	1	2	3	4	5	6	7	8	9	10	11	12
A	no bHRPO		no bHRPO		no bHRPO		no bHRPO		no bHRPO		no bHRPO	
B	no bHRPO		1 / 1		1 / 64		1 / 4096		1 / 262144		no bHRPO	
C	no bHRPO		1 / 2		1 / 128		1 / 8192		1 / 524288		no bHRPO	
D	no bHRPO		1 / 4		1 / 256		1 / 16384		1 / 1048576		no bHRPO	
E	no bHRPO/ABTS		1 / 8		1 / 512		1 / 32768		1 / 2097152		no bHRPO/ABTS	
F	no bHRPO/ABTS		1 / 16		1 / 1024		1 / 65536		1 / 4194304		no bHRPO/ABTS	
G	no bHRPO/ABTS		1 / 32		1 / 2048		1 / 131072		1 / 8388608		no bHRPO/ABTS	
H	no bHRPO/ABTS		no bHRPO/ABTS		no bHRPO/ABTS		no bHRPO/ABTS		no bHRPO/ABTS		no bHRPO/ABTS	

TABLE II

Dilution	Grams bHRPO	Moles bHRPO
1 / 1	2.40E-06	5.33E-11
1 / 2	1.20E-06	2.67E-11
1 / 4	6.00E-07	1.33E-11
1 / 8	3.00E-07	6.67E-12
1 / 16	1.50E-07	3.33E-12
1 / 32	7.50E-08	1.67E-12
1 / 64	3.75E-08	8.33E-13
1 / 128	1.88E-08	4.17E-13
1 / 256	9.38E-09	2.08E-13
1 / 512	4.69E-09	1.04E-13
1 / 1024	2.34E-09	5.21E-14
1 / 2048	1.17E-09	2.60E-14
1 / 4096	5.86E-10	1.30E-14
1 / 8192	2.93E-10	6.51E-15
1 / 16384	1.46E-10	3.26E-15

TABLE II

Dilution	Grams bHRPO	Moles bHRPO
1 / 32768	7.32E-11	1.63E-15
1 / 65536	3.66E-11	8.14E-16
1 / 1E+05	1.83E-11	4.07E-16
1 / 3E+05	9.16E-12	2.03E-16
1 / 5E+05	4.58E-12	1.02E-16
1 / 1E+06	2.29E-12	5.09E-17
1 / 2E+06	1.14E-12	2.54E-17
1 / 4E+06	5.72E-12	1.27E-17
1 / 8E+06	2.85E-13	6.36E-18

After applying statistical curve fitting techniques, a concentration activity curve was generated and is shown in Figure 1. Results are presented in terms of moles of bHRPO. As demonstrated therein, the detection limit of this system approaches femtomolar levels, even using an unamplified system. The typical Gaussian response curve of ELISA is also seen in Figure 1, which further demonstrates that the maximum sensitivity of this assay occurs at bHRPO levels of 2 x 10^{-13} moles.

Example II

Example I demonstrated that immobilized silver microtiter plates are capable of binding biotin and a biotinylated antigen. This example demonstrates that biotinylated capture antibodies can be bound to immobilized silver microtiter plates and used for antigen capture.

Immobilized silver microtiter plates were prepared as described in Example I. The plates were incubated with biotinylated anti-peroxidase antibodies (Jackson Immunological) at an antibody concentration of 1.2 mg/ml diluted 1:100, with addition of 150 microliters to each well for an hour. After washing the plates with 50 mM phosphate buffer with 0.1% v/v Tween 20 detergent (Phosphate/Tween buffer), the enzyme horseradish peroxidase (Sigma) was added, in the amounts shown in

Table III, for one hour incubation. Phosphate/Tween buffer was used as the dilution buffer in the assay.

The results of eight assays of the type shown in Figure 2 are presented in Figure 3, along with 2σ error limits. The data for the immunoassays is shown in Table III, using the eight simultaneous assay replications for a basic statistical analysis. Figure 3 uses kinetic rates to determine the detection of enzyme, which allows for a linear fit of the data when presented on a semi-log plot using the dilution number of the initial enzyme solution. At the initial dilutions, the margin of error is rather high, due to the high amounts of enzyme in the initial solution (2.56×10^{-11} moles per well) producing extremely rapid kinetics. As the enzyme goes through doubling dilutions, the kinetics are measured more easily and the precision of the assay improves at dilution number 16 (1.6×10^{-12} moles per well). The detection limit of this assay is in the region of dilution number 1000 (0.25 femtomoles), which approaches the theoretical detection limit of the enzyme substrate system being used. (Deshpande, Enzyme Immunoassays: From Concept to Product Development, New York, Chapman & Hall (1996), 1-422.

TABLE III

Kinetic Immunoassay Data

Dilution Number	HRPO (g/well)	HRPO (mol/well)	Kinetic Rate	2σ Limits
1	1.13E-06	2.56E-11	927.65	286.73
2	5.63E-07	1.28E-11	773.66	253.20
4	2.81E-07	6.40E-12	666.47	95.75
8	1.41E-07	3.20E-12	598.88	97.22
16	7.03E-08	1.60E-12	551.07	61.58
32	3.52E-08	8.00E-13	452.40	25.79
64	1.76E-08	4.00E-13	361.72	24.07
128	8.79E-09	2.00E-13	277.00	20.58
256	4.39E-09	1.00E-13	193.03	27.47

TABLE III

Kinetic Immunoassay Data

Dilution Number	HRPO (g/well)	HRPO (mol/well)	Kinetic Rate	2σ Limits
512	2.20E-09	5.00E-14	128.36	49.72
1024	1.10E-09	2.50E-14	80.85	53.07
2048	5.49E-10	1.25E-14	65.28	62.02
4096	2.75E-10	6.25E-15	60.26	61.08
8192	1.37E-10	3.12E-15	62.54	60.02
16384	6.87E-11	1.56E-15	52.25	78.54
32768	3.43E-11	7.81E-16	46.92	83.12
65536	1.72E-11	3.91E-16	36.93	69.52
131072	8.58E-12	1.95E-16	40.52	69.90

The basis of the foregoing is the assumption that the enzyme binds only to the antibodies, which specifically select it from the test solution. In order for this to be valid, background binding of the enzyme to the plate must be at insignificant levels. In order to determine this, a checkerboard assay was performed (Deshpande, supra). The assay arrangement is shown in Figure 4. When performed, the initial concentrations of the antibody and the enzyme were the same as described above, as were all buffers and incubation periods. The checkerboard assay, which is primarily visual, is a means to determine the effect of the antibody concentration and the enzyme concentration on the assay results. As seen in Figure 5, the enzyme only binds when antibody is present in the well. As antibody concentrations decrease horizontally across the plate, enzyme binding falls rapidly, as shown by the absence of color on the right side of the plate, even at the extreme enzyme concentrations introduced into row A.

Example III

The foregoing examples demonstrate that immobilized silver is capable of binding biotin in the bHRPO assay and that effective immobilization of capture antibodies in the immobilized silver microplate wells is possible. In the present example, the silver ion immunoassay format is then compared to the current streptavidin technology used to bind biotinylated antibodies. Plates coated with streptavidin (Xenopore, XPS 010 00) were obtained for this purpose. These plates represent the best of the currently available products since they feature a covalent linkage between the streptavidin and the plate surface, and are also blocked with a proprietary nonbiotinylated protein to inhibit background binding. No blocking agents are used on the silver plates. A side by side comparison with an immobilized silver plate prepared according to Example I was performed as follows.

Both the streptavidin plate and the silver plate were hydrated and rinsed with 50 mM pH 7 phosphate buffer. The wells in the plates were filled to capacity with buffer and allowed to stand at room temperature for 10 minutes. The plates were then rinsed twice with the same buffer.

Biotinylated anti-peroxidase antibodies (Jackson Immunological) were used to coat the plates. In this assay, 150 µl of antibody solution (1.2 mg/ml) diluted to 1:100 was added to the wells in the odd numbered columns. The even numbered columns were used as control wells, and were filled with 150 µl of buffer. This arrangement of activated and control wells is shown in Figure 6. The buffer used to dilute the antibodies and fill the control wells was 50 mM pH 7 phosphate buffer with 0.1% v/v Tween 20 Phosphate/Tween) added to inhibit any hydrophobic binding in the plates. The plates were then covered and allowed to stand at room temperature for 1.5 hours.

Both plates were thoroughly washed using a Bio-Rad Plate Washer filled with Phosphate/Tween buffer as the wash buffer. Horseradish peroxidase (Sigma) was used as the antigen in this test. A solution of peroxidase was created by adding 8 x 10^{-4}g of the enzyme to 10 ml of Phosphate/Tween. 1 ml of this solution was then added to 9 ml of buffer. 300 µl of the diluted enzyme solution was added to wells A1, A2, A5, A6, A9, and A10. The amount of enzyme added to these primary wells is shown in Table IV. The remainder of the wells were filled with 150 µl of

Phosphate/Tween. For Test 1, 150 µl of solution was withdrawn from A1 and A2 and diluted into B1 and B2. This procedure was repeated down the plate, with the solution from H1 and H2 carried to wells A3 and A4. Tests 2 and 3 were performed in a similar fashion. After all of these doubling dilutions were complete, the plates were covered and allowed to stand at room temperature for 1 hour.

For enzyme detection the plates were first washed using the automated plate washer with Phosphate/Tween. Then, 150 µl of an ABTS solution was added to each well in the plate, and the rate of formation of the colored product read at 415 nm in a Bio-Rad Benchmark Microplate Reader. Readings took place every 15 seconds for 5 minutes. The ABTS solution was made by adding 17 mg of ABTS to 100 ml of 50 mM pH 5 citrate buffer. Immediately before use, 100 µl of 3% hydrogen peroxide was added to the solution to catalyze the enzymatic reaction.

The raw data for these experiments is presented in Table IV and Table V. Using the average of the normalized tests, a plot comparing the streptavidin plate to the silver plate was prepared. The data points shown on the graph in Figs. 7 are the average values of the normalized tests shown in Tables IV and V. The exception to this are the data points taken at Dilution 1, which were replaced by the values representing the asymptotic approach of the immunoassays as determined from the preceding points. From this, the maximum kinetic rate achievable in the silver plate is 375, versus 150 in the streptavidin plate. Due to the approach to a final value at high levels of enzyme, this difference is due to the amount of capture antibody on the plate. The silver plate binds more functional capture antibody, and thus is able to bind more enzyme when excess enzyme is present. Assuming that each antibody captures an average of 1.5 enzyme molecules, it is possible to estimate that the silver plate has approximately twice as many biotinylated antibody binding sites available. The ultimate detection limits of both plates is the same, however, and approaches femtomolar levels. This is the limit of the enzyme/substrate detection system being used.

TABLE IV

Silver Plate Immunoassay Data

Dilution Number	HRPO (mol/well)	Test 1		Test 2		Test 3		Test 1	Test 2	Test 3	Average
		Antibody	Control	Antibody	Control	Antibody	Control	Normalized	Normalized	Normalized	
1	2.73E-11	2.83E+02	2.52E+01	2.38E+02	1.83E+01	2.90E+02	2.55E+01	2.58E+02	2.20E+02	2.65E+02	2.47E+02
2	1.37E-11	3.87E+02	1.58E+01	3.46E+02	1.15E+01	3.94E+02	1.73E+01	3.71E+02	3.34E+02	3.76E+02	3.61E+02
4	6.83E-12	3.67E+02	9.82E+00	3.76E+02	7.55E+00	3.81E+02	1.22E+01	3.58E+02	3.68E+02	3.69E+02	3.65E+02
8	3.41E-12	3.36E+02	6.04E+00	3.44E+02	4.20E+00	3.63E+02	8.71E+00	3.30E+02	3.40E+02	3.54E+02	3.41E+02
16	1.71E-12	3.07E+02	5.74E+00	2.99E+02	3.37E+00	3.43E+02	7.77E+00	3.01E+02	2.96E+02	3.36E+02	3.11E+02
32	8.53E-13	2.67E+02	1.50E+01	2.70E+02	1.77E+00	2.90E+02	7.32E+00	2.52E+02	2.68E+02	2.83E+02	2.68E+02
64	4.27E-13	2.18E+02	2.83E+00	2.16E+02	2.11E+00	2.28E+02	5.53E+00	2.15E+02	2.14E+02	2.22E+02	2.17E+02
128	2.13E-13	1.61E+02	1.49E+00	1.53E+02	2.30E+00	1.50E+02	7.92E+00	1.60+02	1.51E+02	1.42E+02	1.51E+02
256	1.07E-13	7.38E+01	1.04E+00	7.18E+01	4.99E+00	7.39E+01	2.02E+00	7.27E+01	6.68E+01	7.18E+01	7.05E+01
512	5.33E-14	5.20E+01	1.35E+00	4.73E+01	2.57E+00	5.37E+01	2.32E+00	5.06E+01	4.48E+01	5.14E+01	4.89E+01
1024	2.67E-14	2.81E+01	1.49E+00	2.33E+01	4.83E+00	2.75E+01	3.26E+00	2.67E+01	1.85E+01	2.43E+01	2.31+01
2048	1.33E-14	1.20E+01	1.96E+00	1.34E+01	5.77E+00	1.43E+01	4.13E+00	1.00E+01	7.68E+00	1.02E+01	9.30E+00
4096	6.67E-15	6.66E+00	1.91E+00	1.05E+01	8.35E+00	8.61E+00	5.30E+00	4.75E+00	2.15E+00	3.31E+00	3.41E+00
8192	3.33E-15	4.73E+00	1.49E+00	1.03E+01	8.84E+00	3.03E+00	4.65E+00	3.24E+00	1.43E+00	-1.62E+00	1.02E+00
16384	1.67E-15	2.93E+00	2.41E+00	7.07E+00	6.65E+00	6.78E+00	6.25E+00	5.22E-01	4.28E-01	5.25E-01	4.92E-01
32768	8.33E-16	2.12E+00	3.86E+00	5.24E+00	5.19E+00	6.95E+00	8.48E+00	-1.74E+00	4.82E-02	-1.53E+00	-1.07E+00

TABLE V

Streptavidin Plate Immunoassay Data

Dilution Number	HRPO (mol/well)	Test 1		Test 2		Test 3		Test 1	Test 2	Test 3	Average
		Antibody	Control	Antibody	Control	Antibody	Control	Normalized	Normalized	Normalized	
5	2.73E-11	1.36E+02	6.50E+00	1.33E+02	8.55E+00	1.29E+02	5.50E+00	1.29E+02	1.24E+02	1.24E+02	1.26E+02
2	1.37E-11	1.74E+02	8.61E+00	1.24E+02	3.46E+00	1.75E+02	7.26E+00	1.65E+02	1.20E+02	1.68E+02	1.51E+02
4	6.83E-12	1.74E+02	1.96E+00	1.51E+02	2.29E+00	1.49E+02	2.06E+01	1.72E+02	1.49E+02	1.28E+02	1.50E+02
8	3.41E-12	1.62E+02	1.47E-01	1.34E+02	1.02E+00	1.38E+02	4.83E-01	1.62E+02	1.33E+02	1.38E+02	1.44E+02
16	1.71E-12	1.62E+02	-2.36E-01	1.40E+02	3.03E-02	1.30E+02	-2.53E-01	1.62E+02	1.40E+02	1.30E+02	1.44E+02
10	8.53E-13	1.49E+02	-8.89E-01	1.16E+02	6.89E-01	1.31E+02	-1.41E+00	1.50E+02	1.16E+02	1.33E+02	1.33E+02
64	4.27E-13	1.23E+02	-6.99E-01	1.28E+02	3.23E-01	1.05E+02	-1.26E+00	1.24E+02	1.28E+02	1.06E+02	1.19E+02
128	2.13E-13	1.04E+02	-1.47E-01	1.01E+02	-8.88E-01	8.99E+01	-2.05E+00	1.04E+02	1.02E+02	9.20E+01	9.93E+01
256	1.07E-13	3.86E+01	8.42E-01	5.01E+01	1.06E+00	4.32E+01	5.47E-01	3.78E+01	4.91E+01	4.26E+01	4.32E+01
512	5.33E-14	3.95E+01	1.37E+00	3.31E+01	7.59E-02	4.04E+01	6.97E-02	3.81E+01	3.31E+01	4.03E+01	3.72E+01
1024	2.67E-14	2.45E+01	1.38E+00	2.02E+01	-1.67E-01	2.45E+01	5.45E-01	2.31E+01	2.03E+01	2.40E+01	2.25E+01
2048	1.33E-14	1.29E+01	6.25E-01	9.56E+00	-8.19E-01	1.22E+01	1.06E-01	1.23E+01	1.04E+01	1.21E+01	1.16E+01
4096	6.67E-15	5.97E+00	-3.87E-01	4.76E+00	-1.42E+00	6.07E+00	-3.69E-01	6.36E+00	6.18E+00	6.44E+00	6.33E+00
8192	3.33E-15	2.75E+00	-3.01E-01	1.47E+00	-2.08E+00	3.18E+00	-6.08E-02	3.05E+00	3.55E+00	3.24E+00	3.28E+00
16384	1.67E-15	1.25E+00	-9.64E-01	1.64E-01	-1.36E+00	-2.02E+00	-9.85E-01	2.21E+00	1.52E+00	-1.04E+00	8.97E-01
32768	8.33E-16	1.07E+00	-1.22E+00	-2.82E-01	-1.87E+00	-9.76E-01	-2.00E+00	2.29E+00	1.58E+00	1.02E+00	1.63E+00